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Research Report

Treadmill training improves motor skills and increases tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta in diabetic rats

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ABSTRACT

The aim of this study was to evaluate the effects of treadmill training on motor skills and immunoreactivity to tyrosine hydroxylase in the substantia nigra pars compacta and ventral tegmental area from diabetic rats induced by streptozotocin. Male Wistar rats were divided into three groups: control, diabetic and trained diabetic. Treadmill training was performed for 8 weeks. Blood glucose concentrations and body weight were evaluated 48 h after diabetes induction and every 30 days thereafter. Motor skills were evaluated on the rotarod and open field tests. Then, animals were transcardially perfused and the brains were post-fixed, cryoprotected and sectioned in a cryostat. Immunohistochemistry for tyrosine hydroxylase analyses was done in the ventral tegmental area and in the substantia nigra. Motor skills showed that diabetic animals had a decrease in the latency to fall and enhanced number of falls in the rotarod test compared to control and trained diabetic animals. In the open field, diabetic animals had a decrease in the number of crossed squares, rearings and spent a less time moving compared to control and trained diabetic animals. In diabetic animals, optical densitometry of immunohistochemistry showed that tyrosine hydroxylase reaction decreased in the ventral tegmental area and in the neurons and process in the substantia nigra. In the later region, that decrease was reversed by treadmill training. In conclusion, we demonstrated that treadmill training can reverse the loss of the motor skills, which was correlated to tyrosine hydroxylase immunoreactivity in the substantia nigra of diabetic animals without pharmacological treatment.

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1. Introduction

Diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, heart, blood vessels and nerve fibers (American Diabetes Association, 2010a). Diabetic neuropathy is highly prevalent and causes particularly significant morbidity to affected patients (Tesfaye et al., 2010). Moreover, streptozotocin (STZ)-induced diabetes in rats causes degenerative changes in the autonomic nervous system (Schaan et al., 2004), sensory neurons (Sidenius and Jakobsen, 1980; Fernyhough et al., 1999; Zherebitskaya et al., 2009; do Nascimento et al., 2010), and brain structures, such as the cerebellum (Anu et al., 2010) and the substantia nigra pars compacta (SN_{pc}; Figlewicz et al., 1996), causing deficits in the autonomic, sensory and motor systems.

The SN_{pc} and the ventral tegmental area (VTA) are motor structures that provide largely dopaminergic inputs to the cortex, striatum and to a lesser extent, pallidum (Paxinos, 1995). These structures are vulnerable to damage caused by exogenous toxins (McCormack et al., 2004), by aging, causing motor impairment (Emborg et al., 1998; Stark and Pakkenberg, 2004), and also by hyperglycemia of diabetes in rats (Figlewicz et al., 1996). Moreover, tyrosine hydroxylase (TH), which catalyzes the conversion of L-tyrosine to L-dopa and is the initial and rate-limiting step in the biosynthesis of catecholamines, has been used for the study of dopaminergic neurons (Nakashima et al., 2009).

Although the beneficial effects of regular physical exercise are well-known and used as part of the treatment of diabetic patients (American Diabetes Association, 2010b), few data on its efficacy in human diabetic neuropathy have been reported (Balducci et al., 2006). In addition, some studies in rats have shown the benefits of treadmill training in diabetes-induced cardiovascular and autonomic dysfunction (De Angelis et al., 2000; Harthmann et al., 2007), as well as in sensory neuropathy (do Nascimento et al., 2010). However, there are no data available on the effectiveness of treadmill training on motor deficits caused by diabetes in animals. Thus, the aim of this study was to evaluate the effects of a treadmill training protocol on motor skills and immunoreactivity to tyrosine hydroxylase (TH-ir) in the SN_{pc} and ventral tegmental area (VTA) of rats with STZ-induced diabetes.

2. Results

2.1. Body weight and blood glucose concentrations

There were no differences in the body weight between the C (298±5.1), D (295±4.6) and TD (305.8±6.5) groups 48 h before diabetes induction ($P>0.05$). Moreover, 30, 60 and 90 days after diabetes induction, rats from the D (253.3±16.7; 238±16; 237.7±15.7 respectively) and TD groups (281.3±5.6; 269.7±9; 277.7±11 respectively) showed lower body weight than the C group (351.3±3.9; 383.7±3.2; 406±2.9 respectively; $P<0.001$; Table 1).

As expected, 48 h after diabetes induction, blood glucose was higher in the diabetic groups (D and TD; 380.2±22.1 and 365.2±17.1 respectively) vs. the C group (86.3±4.6; $P<0.001$). In addition, the D and TD groups showed higher blood glucose than C group 30 (526±23.5; 485.1±37.3; 89.8±2.5 respectively; $P<0.001$), 60 (521.5±11.5; 512±17.6; 88.8±2.2 respectively; $P<0.001$) and 90 (514.7±18.7; 500.7±22.4; 94±2.7 respectively; $P<0.001$) days later. However, there were no differences between the D and TD groups in any of these variables ($P>0.05$; Table 1).

2.2. Motor skills

2.2.1. Rotarod test

Animals from group D presented a lower latency to fall (37.5±3.2) as compared to those in the C (56.6±1.7; $P<0.001$) and TD groups (53.4±2.3; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$; Fig. 1a). In addition, the D group (4.2±0.3) was seen to fall more frequently than the C (0.8±0.3; $P<0.001$) and TD (1.7±0.5; $P<0.001$) groups. However, there were no differences between the C and TD groups ($P>0.05$; Fig. 1b).

2.2.2. Open field test

The number of squares crossed by animals from the D group (10.1±1.4) was lower than in the C (22.1±3.5; $P<0.05$) and TD groups (29.4±3.9; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$; Fig. 2a). Furthermore, in the open field, the D group spent less time (15.3±2.4) moving than the C (33.7±3.1; $P<0.05$) and TD groups (34.2±4.8; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$;

Table 1 – Time course changes in body weight and blood glucose in the studied rats.

Body weight and blood glucose in the studied groups								
Group	48 h		30 days		60 days		90 days	
	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)
C	298.0±5.1	86.3±4.6	351.3±3.9	89.8±2.5	383.7±3.2	88.8±2.2	406±2.9	94±2.7
D	295.0±4.6	380.2±22.1*	253.3±16.7*	526.0±23.5*	238.0±16*	521.5±11.5*	237.7±15.7*	514.7±18.7*
TD	305.8±6.5	365.2±17.1*	281.3±5.6*	485.1±37.3*	269.7±9*	512.0±17.6*	277.7±11*	500.7±22.4*

C: control group; D: diabetic group; TD: trained diabetic group. Body weight: repeated measures ANOVA group effect [$F_{(2,15)}=48.208$; $P<0.001$], time effect [$F_{(3,45)}=42.702$; $P<0.001$], time vs. group interaction [$F_{(6,45)}=42.702$; $P<0.001$]. Glycemia: repeated measures ANOVA group effect [$F_{(2,15)}=405.504$; $P<0.001$], time effect [$F_{(3,45)}=22.611$; $P<0.001$], time vs. group interaction [$F_{(6,45)}=5.313$; $P<0.001$].
* corresponds to $P<0.001$ compared to the C group.

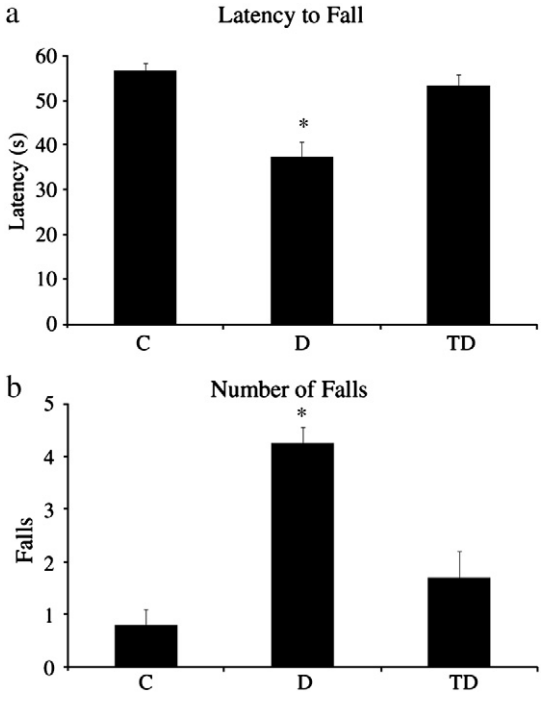

Fig. 1 – a: Latency to fall in the rotarod test. One way ANOVA [$P<0.001$; $F_{(2,25)}=16, 548$] with Bonferroni *post hoc* test. * corresponds to $P<0.001$ compared to the C and TD groups. b: Number of falls in the rotarod test. One way ANOVA [$P<0.001$; $F_{(2,25)}=19, 70,120$] with Bonferroni *post hoc* test. * corresponds to $P<0.001$ compared to the C and TD groups.

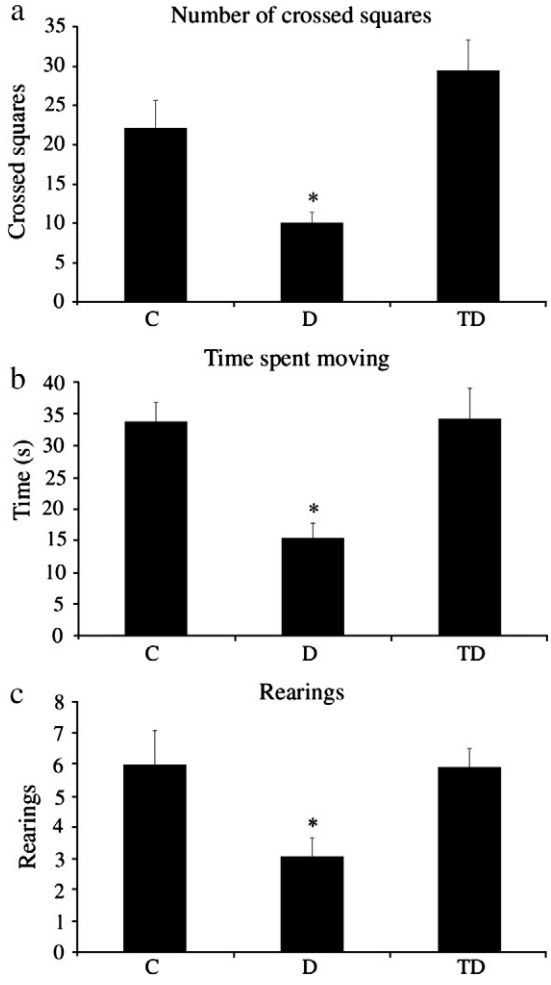

Fig. 2 – a: Number of crossed squares in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=11, 9815$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups. b: Mean time spent moving in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=9, 8645$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups. c: Number of rearings in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=5, 3100$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups.

Fig. 2b). The D group was seen to rear (3.1±0.6) less frequently than the C (6.0±1.1; $P<0.05$) and TD (5.9±0.6; $P<0.05$) groups. There were no differences between the C and TD groups ($P>0.05$; Fig. 2c).

2.3. *Optical densitometry of TH-ir*

The OD analysis of the VTA showed that the TH-ir was lower in the neurons and processes from the D group (0.44±0.01) than in group C (0.51±0.01; $P<0.05$). However, there were no differences between the TD (0.5±0.02) and C groups ($P=1.0$), or between the TD and D groups ($P=0.08$; Fig. 3a).

Interestingly, the OD analysis of the SN_{pc} showed that the TH-ir of neurons and processes in the D group (0.35±0.01) was lower than in the C (0.42±0.01; $P<0.05$) and TD groups (0.43±0.01; $P<0.05$). However, there were no differences between C and TD groups ($P>0.05$; Fig. 3b). Images from the groups are shown in Fig. 3c.

3. Discussion

The present study showed that treadmill training alone, with no pharmacological intervention, can reverse the loss of motor skills previously induced by STZ in rats, an improvement that was associated with tyrosine hydroxylase immunoreactivity changes in the substantia nigra and ventral tegmental area.

As expected, diabetic rats induced by STZ displayed higher blood glucose levels and lower body weights when compared to control animals. The treadmill training did not reduce blood glucose nor body weights, which is in accordance with previous results from our (do Nascimento et al., 2010) and other group (Midaoui et al., 2006), showing that physical training alone is not able to significantly improve metabolic control in these animals.

In the rotarod test, diabetic animals performed less well than the control and trained diabetic animals, showing lower latency to fall and a greater number of falls during the test. This task tests locomotion and coordination (Dunham and Miya, 1957); thus, it is evident that diabetic animals had a decrease in the motor coordination, affecting motor systems, as previously shown (Peeyush et al., 2009; Abraham et al., 2010). Interestingly, trained diabetics performed as well as

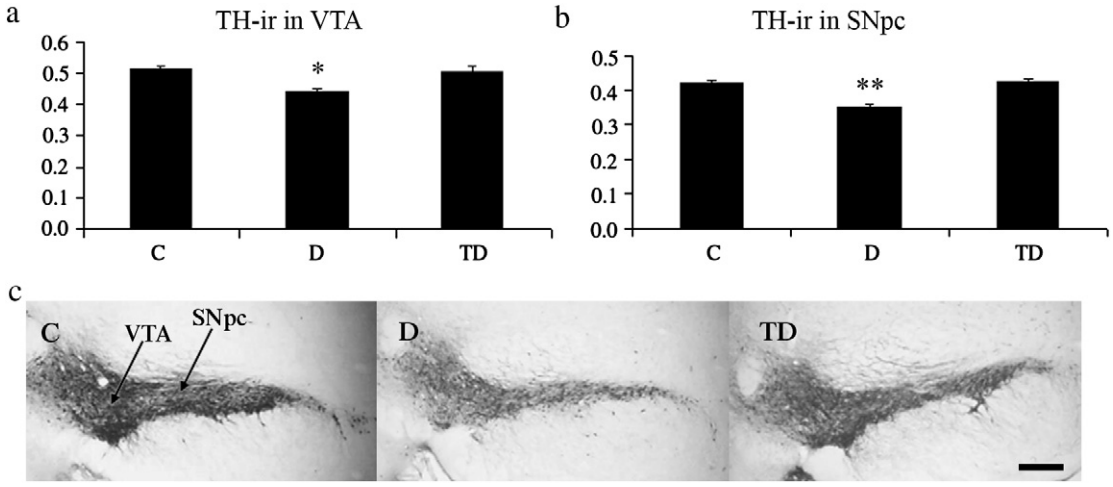

Fig. 3 – a: TH immunoreactivity (TH-ir) in the ventral tegmental area (VTA). One way ANOVA [$P<0.05$; $F_{(2,15)}=4,681$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C group. b: TH immunoreactivity (TH-ir) in the substantia nigra pars compacta (SN_{pc}). One way ANOVA [$P<0.001$; $F_{(2,15)}=8,912$] with Bonferroni *post hoc* test. ** corresponds to $P<0.05$ compared to the C and TD groups. c: Digitalized images of coronal sections of substantia nigra pars compacta (SN_{pc}) and ventral tegmental area (VTA) in C, D and TD animals showing the TH immunoreactivity in neurons and processes. Note the decreased immunoreaction in the VTA and SN_{pc} from the D animal. In the image from the TD animal, note the lower immunoreaction in the VTA, compared to C animal, and the immunoreaction in the SN_{pc} which is similar to the C animal. Scale bar: 300 μm.

nondiabetic rats in this test, showing that exercise was able to reverse motor dysfunction and coordination deficits determined by diabetes, a finding not described before.

In the open field task, diabetic animals were seen to spend less time moving, crossed fewer squares and reared less frequently than the animals in the C and TD groups. All of these results demonstrate that diabetic animals were bradykinetics, resulting in a less exploratory behavior. Our results from both motor tasks, as well as the modification in the TH-ir from neurons and processes of SN_{pc} in STZ-diabetic rats suggest the involvement of the motor centers of the brain in the altered motor activity.

Additionally, in our study, the diabetic animals were seen to have a lower TH-ir in the VTA, probably giving rise to lower production of dopamine. However, although treadmill training improved motor skills, it was unable to reverse the decrease in TH-ir in the VTA. Moreover, the VTA plays a central role in multiple critical brain functions, including cognition, motivation, reward (Nieoullon, 2002; Wise, 2004; Fields et al., 2007) and together with the SN_{pc} influences locomotor activity (Paxinos, 1995; Schultz, 2007). However, there are differences in the morphological and electrophysiological properties of the dopaminergic neurons in these two regions, such as in the ionic channels (Neuhoff et al., 2002; Khaliq and Bean, 2010), which can cause different responses to injury and physical activity. In addition, although the treadmill training did not completely reverse the decrease in the VTA-ir, there was a strong trend toward normal values.

The SN_{pc} provides dopaminergic inputs to the cortex, striatum and pallidum, which facilitate most loops and outputs in the extrapyramidal motor system (Paxinos, 1995). However, the untrained diabetic rats had lower TH-ir in the SN_{pc}, which is in agreement with a previous study, in which diabetic animals were found to have lower TH mRNA levels in the SN_{pc}/VTA (Figlewicz et al., 1996). This decrease in TH reaction could be explained by changes in the total number of cells, in the total number of immunoreactive cells, in the immunostained area and/or by changes in intracellular immunoreactivity, as observed in an animal model of Parkinson's disease (Xavier et al., 2005). Interestingly, hyperglycemia causes oxidative stress and mitochondrial dysfunction (Mastrocola et al., 2005), leading to vascular damage and consequently hypoxia in the brain (Muresanu et al., 2010), which may contribute to neuronal death or reduced dopamine production, causing a decrease in the TH-ir in neurons and processes. Thus, in our study, STZ-diabetic rats presented motor alterations that were modified by treadmill training which recuperates TH-ir in the SN_{pc}, contributing to the maintenance of the extrapyramidal motor system of these rats.

On the other hand, brain derived neurotrophic factor (BDNF) is a neurotrophin that is enhanced by physical exercise in the hippocampus and is associated with the object recognition memory (Hopkins and Bucci, 2010) and improvement in the spatial memory (Khabour et al., 2010). Exercise alters the BDNF expression in the spinal cord of adult rats (Macias et al., 2007), in the cerebellum and motor cortex (Klintsova et al., 2004). In addition, BDNF also regulates early postnatal cell death in the SN_{pc} (Oo et al., 2009), and exercise exerts a neuroprotective effect in an animal model of Parkinson's disease (Yoon et al., 2007; Tajiri et al., 2010). Given this, we hypothesized that the improvement in the motor skills and in the TH-ir provided by the treadmill training in the STZ-diabetic rats could be caused by the BDNF downstream effects.

3.1. *Conclusions*

In summary, our results show that diabetes induced by STZ causes motor abnormalities and reduced TH-ir in the SN_{pc}.

Treadmill training promotes an increase in motor skills and behavior, which is accompanied by changes in TH-ir in the SN_{pc}, but not in the VTA.

4. Experimental procedures

4.1. Animals

Thirty three male Wistar rats (12 weeks old) from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul) were housed under standard laboratory conditions with food and water available *ad libitum* and maintained under a 12:12 light/dark cycle (lights on at 8:00 h). All efforts were made to minimize the number of animals studied and their suffering. The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires, and the International Brain Research Organization, and in compliance with the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). This study was previously approved by the Ethical Committee from UFRGS under the protocol number 2008-062.

4.2. Experimental design

The rats were divided in three groups as follows: non-diabetic rats (C), diabetic rats (D) and diabetic rats submitted to treadmill training (TD). For analyses of motor skill in the rotarod, 10 animals were used in group C, 8 animals in group D and 10 animals in group TD. In the open field, 9 animals were used in group C, 13 animals in group D and 11 animals in group TD. Six animals per group were randomly selected for immunohistochemistry studies.

4.3. Diabetes induction

After an overnight fasting period (6 h), the rats received a single intravenous injection of STZ (50 mg/kg of body weight; Sigma Chemical Co., USA) diluted in 10 mM citrate buffer, pH 4.5. Non-diabetic animals received only citrate buffer (Junod et al., 1969; do Nascimento et al., 2010). Blood glucose concentrations were evaluated in blood collected from the rat-tail using test strips (Performa, Roche, Indianapolis, USA). Diabetes was defined as a fasting glucose >300 mg/dL in tail vein blood 48 h after STZ injection (Junod et al., 1969). Body weights and blood glucose concentrations were measured 48 h after the induction of diabetes and every 30 days thereafter.

4.4. Maximal exercise test

At the 4th week after diabetes induction, all animals underwent adaptation to a treadmill originally designed for human use (Runner, Brazil) and modified for use in rats during 10 minutes at 5 m/min for 4 days. On the 5th day, the rats were submitted to a maximal exercise test (MET), consisting of a graded exercise on the treadmill, with speed increments of 5 m/min every 3 minutes, starting at 5 m/min and continuing up to the maximal intensity attained by each rat, and was stopped when each animal remained more than 50% of the time without giving signs of intention to advance (Melo et al., 2003; Rodrigues et al., 2007; Ilha et al., 2008; do Nascimento et al., 2010). The values obtained in the MET were used to plan the treadmill training program, which started in the 5th week after diabetes induction. In order to correct the exercise intensity, a second MET was performed in the fifth training week.

4.5. Treadmill training

Exercise was performed on a treadmill twice a day, with an interval of 4 h between each session, 5 days per week (Tancrède et al., 1982), and the training intensity increased gradually, according to the MET results. During the first week, the running sessions lasted 10 min, and the duration of each increased each week, reaching 60 min in the 7th week, which was maintained until the 8th week. Moreover, each training session consisted of a warm-up period, a main period and a cooling-off period. During the warm up period, the rats ran 15% of the session time at 30% of the maximum velocity determined by the MET; in the main period, the rats ran 70% of the session time at 60% of the maximum velocity; and in the cooling-off period, the rats ran 15% of the session time at 30% of the maximum MET values.

4.6. Motor skills

4.6.1. Rotarod test

On the day after the last session of treadmill training, the rats were trained to remain on the rota rod apparatus (Insight, Brazil) with the speed adjusted to 12 rpm for 60 s. The following day, the selected rats were tested in the apparatus with the speed adjusted to 16 rpm for 5 sixty-second trials (modified from Linck et al., 2009). The latency to fall (data presented as the mean of the 5 trials) and the number of falls were evaluated.

4.6.2. Open field test

The rats were gently placed in the corner of a 40 cm × 50 cm × 60 cm box, in which the floor was divided into 12 squares, and then filmed with a digital camcorder (DCR-SR47, Sony, Japan) for 3 min (modified from Moreira et al., 2010). The number of crossings from one square to another, the time spent moving, and the number of rearings were counted.

4.7. Immunohistochemical procedure

One day after the analyses of the motor skills, rats were anesthetized with sodium thiopental (i.p; 50 mg/kg; Cristalia, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle, then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Brazil, 20 mL/min) with 400 mL of 0.9% saline solution, followed by 400 mL of a fixative solution 4% paraformaldehyde (Synth, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed

from the skulls, post-fixed in the same solution at room temperature for 4 h and cryoprotected by immersion in a 15% and 30% sucrose (Synth, Brazil) solution in PB at 4 °C until they sank. After these procedures, the brains were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and kept in a freezer (–70 °C) for further analyses.

Coronal sections (50 μm) from VTA and SN_{pc} were obtained from each brain using a cryostat (CM1850, Leica, Germany) at –20 °C and collected in a PB saline (PBS), pH 7.4. These areas were identified using Paxinos and Watson's Atlas (1998). The free-floating sections were pre-treated with 3% hydrogen peroxide for 30 min, carefully washed and treated with 2% bovine serum albumin (Inlab, Brazil) in PBS containing 0.4% Triton X-100 (PBS-Tx) for 30 min and incubated with monoclonal TH antibody (Sigma Chemical Co., USA) raised in mice, diluted 1:2000 in PBS-Tx for 48 h at 4 °C.

Sections were again washed in PBS-Tx and incubated in an anti-mouse antibody conjugated with peroxidase (Sigma Chemical Co., USA) diluted 1:200 in PBS-Tx for 2 h at room temperature. The reaction was revealed in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and then 1 μL of 3% H_2O_2/mL was added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Germany) and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS.

4.8. *Optical densitometry*

Semi-quantitative densitometric analysis was used to measure the intensity of the TH immunoreaction using a Nikon Optiphot-2 microscope (40×, Japan) coupled to a Micrometrics camera (Accu Scope, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, USA). The digitized images obtained from the selected areas were converted to an 8-bit gray scale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares measuring 9680 μm^2 (area of interest, AOI) overlaid on the gray scale image. Both left and right sides of each brain were used. For each rat, 10 measures were taken from the VTA and 10 measures each from the medial, lateral and intermediary regions of the SN_{pc}. The results shown for the SN_{pc} were the total mean value from the three studied regions.

Background staining subtraction and correction were done in accordance with our previous published protocol (Xavier et al., 2005).

The optical density (OD) was calculated using the following formula:

$$OD(x,y) = -\log[(INT(x,y)-BL)] / (INC-BL)$$

Where "OD(x,y)" is the optical density at pixel(x,y), "INT(x,y)" or intensity is the intensity at pixel(x,y), "BL" or black is the intensity generated when no light goes through the material and "INC" is the intensity of the incidental light.

4.9. *Statistical analysis*

Blood glucose and body weight data were analyzed using repeated measures analysis of variance (ANOVA), and differences between the groups were assessed using the Bonferroni *post-hoc* test. Data obtained from motor skills tests, as well as optical densitometry of TH-ir were analyzed using one-way ANOVA and Bonferroni *post-hoc* test. Statistical significance was set at $P<0.05$. Data were run on Statistica 6.0 software package (StatSoft, Inc., USA). All data are represented by the mean ± standard error of mean (SEM).

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